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Comparative study of anticoagulant and procoagulant properties of 28 snake venoms from families Elapidae, Viperidae, and purified Russell's viper venom-factor X activator (RVV-X)

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ABSTRACT

Snake venoms consist of numerous molecules with diverse biological functions used for capturing prey. Each component of venom has a specific target, and alters the biological function of its target. Once these molecules are identified, characterized, and cloned; they could have medical applications. The activated clotting time (ACT) and clot rate were used for screening procoagulant and anticoagulant properties of 28 snake venoms. Crude venoms from *Daboia russellii siamensis*, *Bothrops asper*, *Bothrops moojeni*, and one *Crotalus oreganus helleri* from Wrightwood, CA, had procoagulant activity. These venoms induced a significant shortening of the ACT and showed a significant increase in the clot rate when compared to the negative control. Factor X activator activity was also measured in 28 venoms, and *D. r. siamensis* venom was 5–6 times higher than those of *B. asper*, *B. moojeni*, and *C. o. helleri* from Wrightwood County. Russell's viper venom-factor X activator (RVV-X) was purified from *D. r. siamensis* venom, and then procoagulant activity was evaluated by the ACT and clot rate. Other venoms, *Crotalus atrox* and two *Naja pallida*, had anticoagulant activity. A significant increase in the ACT and a significant decrease in the clot rate were observed after the addition of these venoms; therefore, the venoms were considered to have anticoagulant activity. Venoms from the same species did not always have the same ACT and clot rate profiles, but the profiles were an excellent way to identify procoagulant and anticoagulant activities in snake venoms.

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1. Introduction

There are more than 300 species of venomous snakes in the world, and their venoms have numerous molecules with diverse biomedical functions. Each component in the venom has different target and alters biological functions of the target's cells or molecules in various reactions. Disintegrins target platelets, hemorrhagic proteinases target basement membranes, fibrinogenases target fibrinogen, and procoagulants and anticoagulants target the clotting cascade. Snake venoms are excellent sources of molecules for drug discovery, and once purified, characterized, and cloned, could have potential applications in medicine.

Snake procoagulant molecules, especially from the Viperidae family, have been used in medical applications and as diagnostic tools. A procoagulant protein, Batroxobin from *Bothrops atrox* is useful for fibrinogen level assays (Reptilase™ time) and fibrinogen degradation products (FDPs) detection (Aronson, 1976; Stocker and Barlow, 1976;

Abbreviations: ACT, activated clotting time; ET, electrophoretic titration; pI, isoelectric point; RVV, Russell's viper venom; RVV-X, Russell's viper venom-factor X activator; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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Johnson et al., 1977; Hutton and Warrell, 1993; Bell, 1997; Van Cott et al., 2002). Another known procoagulant from Russell's viper venom (RVV), RVV-factor X activator (RVV-X) is useful for measuring a lupus anticoagulant (Thiagarajan et al., 1986; Lo et al., 1989; Derksen and de Groot, 2004).

Procoagulant and anticoagulant properties are widely studied from the Viperidae family. Only a few anticoagulants have been isolated from snake venoms of the Elapidae family (Kini and Evans, 1991; Sundell et al., 2003; White, 2005; Gowda et al., 2006; Kumar et al., 2010).

In this report, we studied procoagulant and anticoagulants of 28 different snake venoms from nine species in two different families, Viperidae and Elapidae, using the Sonoclot assay and then compared them to purified RVV-X from crude *Daboia russellii siamensis*. Their activated clotting time (ACT) and clot rate signatures were determined.

2. Materials and methods

2.1. Venom

The National Natural Toxins Research Center (NNTRC) at Texas A&M University-Kingsville, Kingsville, TX, provided crude venoms from one *Bothrops asper*, one *Bothrops moojeni*, four *Crotalus adamanteus*, three *Crotalus atrox*, five *Crotalus horridus*, five *Crotalus oreganus helleri*, two *Naja melanoleuca*, and six *Naja pallida*. *D. r. siamensis* venom was obtained from the Queen Saovabha Memorial Institute (QSMI, Thai Red Cross Society, Bangkok) and was pooled venom from an underdetermined number of snakes. All snake venoms provided by the NNTRC were identified by their avid number and were never pooled. The avid number refers to individual snake venom. The geographical locations and avid numbers of snake venoms are listed in Table 2. The avid numbers are listed so that the same venoms could be used in future studies. Additional information about the snakes can be found on the NNTRC homepage (http://ntrc.tamuk.edu) by querying the snake venom by its avid number.

2.2. Purification of RVV-X

RVV-X was purified from crude *D. r. siamensis* venom by a modification of the procedure of Kisiel et al. (1976). Briefly, samples of 125 mg of lyophilized crude *D. r. siamensis* venom were mixed with 5.0 mL of 0.1 M sodium phosphate buffer, pH 7.5 containing 1 mM benzamidine–HCl. Two hundred microliters of clear supernatant, at a concentration of 25 mg/mL, were applied into a Waters PROTEIN-PAK™ 300SW (7.5 × 300 mm) HPLC column. The column was previously equilibrated with the elution buffer (0.1 M sodium phosphate buffer, pH 7.5 containing 1 mM benzamidine). The collection process required 60 min, with a flow rate of 0.5 mL/min. A Waters 2487 Dual λ Absorbance Detector was used to monitor absorbencies at 280 nm. Each fraction was screened for factor X activator activity using chromogenic substrate S-2765. The molecular weight and protein patterns of each fraction were determined by SDS-PAGE. Fraction 3 samples had the highest molecular weight proteins with factor X activator activity (Fig. 1a) and had isoelectric point (pI) in the pH range 4–6 as determined by the Electrophoretic titration (ET) (Fig. 1b). The pooled fraction 3 peaks were dialyzed in 0.02 M Tris–HCl buffer, pH 8.0 and further purified by a DEAE anion exchange HPLC chromatography.

The optimal conditions for the second step of purification were determined by the ET curve using a Pharmacia Biotech PhastSystem (Perez et al., 2001). Two hundred microliters of the pooled venom fraction 3 at the concentration of 31.93 mg/mL were applied into a Waters PROTEIN-PAK™ 5PW (7.5 × 75 mm) HPLC column, which was previously equilibrated with 0.02 M Tris–HCl buffer, pH 8.0. The fractions were eluted using 0.02 M Tris–HCl buffer, pH 8.0 with a 0–0.5 M NaCl salt gradient. The collection required 60 min with a flow rate of 1 mL/min. A Waters 2487 Dual λ absorbance detector was used to monitor absorbencies at 280 nm. Breeze software was used to control the pumps and store data. Fractions of various volumes were collected and retention times of the fractions were recorded. Each fraction was screened for factor X activator activity using chromogenic substrate S-2765. Two anion exchange runs were made and fraction 5 samples with factor X activator activity were pooled. The molecular weight and purity of purified RVV-X were determined by SDS-PAGE and verified by mass spectrometry, which was carried out by the laboratory for Biological Mass Spectrometry, Texas A&M University, College Station, Texas. The ET curve was used to predict pI of purified RVV-X.

2.3. Human normal citrated plasma

Pooled normal citrated plasma was prepared by taking samples from five healthy human donors. From each donor, thirty-six milliliters of blood was drawn with a butterfly needle attached to 19 gauge 3/4″ long. Blood was collected by gravity into a 50 mL plastic test tube containing 4 mL of 3.2% sodium citrate. After the blood was drawn, the tube was inverted gently twice to ensure full citration of blood. The citrated blood was centrifuged at 2000 g for 20 min at 25 °C. The plasma was standardized by pooling the plasma from different individuals and 0.7 mL was aliquoted into 1.5 mL plastic tubes, and frozen immediately at −80 °C.

2.4. Procoagulant and anticoagulant activities

The procoagulant and anticoagulant activities of *B. asper*, *B. moojeni*, *C. adamanteus*, *C. atrox*, *C. horridus*, *C. o. helleri*, *D. r. siamensis*, *N. melanoleuca*, *N. pallida*, and purified RVV-X from *D. r. siamensis* were measured using the Sonoclot analyzer, which measures the activated clotting time (ACT) and clot rate by measuring viscosity changes of a whole blood or plasma sample (von Kaulla et al., 1975). The time that plasma remains a liquid is reported as the ACT. The clot rate is defined as the rate of fibrin polymerization, which is the slope in the linear part of the curves in Fig. 2 and is defined as the change clot signal with change in time ($U = \Delta\text{signal}/\Delta\text{time}$). A cuvette was placed into the cuvette holder, which maintains the temperature at 37 °C. A pre-warmed 10 μL sample (37 °C) of a 0.3 M $CaCl_2$ was added to one side of the cuvette. A 10 μL sample of each venom at a concentration of 1 μg/mL or a 10 μL of purified